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for Breast Cancer Cells

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13. ABSTRACT (Maximum 200 Words)

Applying a functional strategy, starting with the transfer of an intact chromosome 16 into breast tumor cells, we identified a replicative senescence gene, SEN16, at 16q24.3. Positional information led to the isolation of a BAC clone that restores normal growth pattern and senescence in immortal breast tumor cells. The nucleotide sequences of cDNA clones, located on the BAC, were compared to recognize overlapping clones. Four full-length cDNAs, representing three genes, were assembled from the sequence of partial cDNAs and cloned into a mammalian cell expression vector. Ectopic expression of one of the full-length cDNA led to growth inhibition and senescence in breast cancer cells. *In silico* analysis of the gene revealed that predicted protein is a part of an ubiquitin-ligase complex involved in protein degradation pathway. Malfunctioning of an ubiquitin dependent protein degradation pathway may contribute to the development of cancer through the deregulation of cell cycle control mechanisms. Our data show that SEN16 is a growth regulatory gene involved in the etiology of breast cancer. Further characterization of SEN16, for the identification of signaling pathways will afford insight into the regulation of cell growth and senescence, which eventually may lead to the development of new diagnostic and therapeutic strategies.

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Annual report

A. Introduction

Normal diploid mammalian cells display a limited proliferative life span in culture (1-3). At the end of the proliferative phase, cells enter a state of irreversible post mitotic growth arrest called senescence (1-3). Mouse hyperplastic alveolar nodule (HAN) cells can be passaged indefinitely from animal to animal by implanting in mouse mammary fat pads and are at high risk for tumor development. In contrast, normal mammary epithelial cells form normal ductal tree cannot be passaged for more than 3-5 times (4). Spontaneous escape from senescence can occur in most rodent cells (5) but has never been reported in human cells. Normal human cells can be transformed by exposure to radiations (6), oncogenic viruses (7) or chemical carcinogens (8), but additional genetic alterations are required to acquire indefinite proliferation (9, 10). Immortalization, therefore, appears to increase the susceptibility to malignant transformation, while cell senescence protects the organism against the consequences of deregulated cell growth (11).

Although a number of molecular differences between immortal and senescent cells have been observed, the changes in gene expression which induce cellular senescence and which result from the onset of senescent program are not known (reviewed in 12). While abrogation of p53 and pRb expression allows normal human fibroblasts to grow for an additional 20-30 doublings, inhibitors of cyclin dependent protein kinases (CDKs), p21 and p16, are over expressed in senescent cells. Serum stimulated senescent cells remain at the G1 restriction point and fail to induce G1 genes but are resistant to apoptosis (13). The well documented progressive loss of telomere length due to diminished telomerase activity in normal cells, but not in immortal cell lines, is an attractive candidate for a "molecular clock" that limits cell proliferation to a finite number of replications (14, 15). However, this correlation is not absolute (16).

In somatic cell hybrids between normal and immortal cells, cellular senescence is expressed as a dominant phenotype over indefinite proliferation (17, 18). By the transfer of individual human chromosomes into a variety of immortal cell lines, cell senescence genes have been mapped on at least ten different chromosomes (19-26). Although several different chromosomes were found to restore normal cell growth and senescence in breast cancer cells, we chose to focus on chromosome 16, because abnormalities in 16q have been repeatedly documented in breast as well as in other tumors (27). Successful positional cloning strategies have been devised to isolate disease genes using linkage analysis and chromosome rearrangements to identify the locus. However, these strategies cannot be applied to the cloning of cellular senescence genes because these cannot be identified in LOH studies or tracked by linkage analysis. To overcome these difficulties, we have devised a 'Functional-Positional Cloning' approach (28-29). Starting with intact chromosomes, using functional criteria to track the gene, we mapped a cell senescence gene, SEN16, within 2-3cM genetic interval at 16q24.3 (29). Here we propose to clone senescence gene, SEN16 gene and study its role in the development of breast cancer.

B. Body

Specific Aims of the original Proposal

Aim 1:	a.	Isolation of expressed sequences for BAC 346J21
	b.	Identification of candidate cDNA clones
Aim 2:	a.	Isolation of full-length cDNA clones
	b.	Functional analysis of full-length cDNA clones.
	c.	Structural analysis of Senescence Gen
Aim 3:		Analysis for Mutational Inactivation of SEN16
Aim 4:		Analysis for the Expression of SEN16 in immortal breast tumor & normal cells.

Results:

1. Identification of a YAC clone carrying SEN16

We have applied a combination of functional complementation and positional cloning strategy to map SEN16 to 16q24.3. Introduction of an intact normal human chromosome 16 into human (MCF.7) and rat (LA7) mammary tumor cell lines restored normal cell growth and senescence. While chromosome transfer clones were maintained in selection medium, sporadic immortal revertant clones arose among senescent cells. Reversion to immortal growth could occur due to inactivation of the senescence gene, either by a mutation or deletion. Analysis of revertant clones, for the deletion of chromosome 16 specific DNA markers, revealed a consensus deletion, spanning a genetic interval of approximately 3-7 cM at 16q24.3 (Reddy et. al. 1999, Oncogene 18:5100-5107). Twenty-four STS markers, mapping in the region of senescence activity, were used to identify corresponding Yeast Artificial Chromosome (YAC) clones spanning this region. YAC clones were retrofitted to incorporate a selectable marker *neo* for selection of mammalian cells in G418. Introduction of retrofitted YACs into human (MCF.7) and rat (LA7) immortal mammary tumor cells identified a 360Kb YAC that conferred senescence in mammary tumor cells (Reddy et. al. 2000, Oncogene 19: 217-222).

2. Identification of a BAC clone carrying SEN16

To further narrow down the position of SEN16, PCR ready DNA pools of a human BAC library (Research Genetics Inc.), comprising 150,000 clones, were screened with 25 STS markers that map in the vicinity of the SEN16 locus. Additional BAC clones, corresponding to mapped markers, were also identified by a search of the human genome database. In order to eliminate false positives, each of the 35 BACs, thus identified, was analyzed for the presence of the query marker as well as other STS markers mapped in the consensus region of SEN16 locus. All BAC insert ends were sequenced using universal primers to the T7 and SP6 vector sites. PCR primers, designed from the BAC ends, were used to identify the overlapping BAC clones by PCR. These data allowed us to assemble a BAC contig.

In order to fill in gaps in the contig map, the human BAC library was re-screened with primers from BAC ends, flanking each gap. BACs thus identified were further analyzed for the presence of other mapped markers. These data allowed us to construct a detailed contig map, comprised 15 BACs, for a 1.8 Mb DNA segment (Fig. 1A). Each BAC clone was sized by Pulsed Field Gel electrophoresis and assigned a fingerprint following digestion with HindIII (data not shown). The relative order of BAC clones in the contig was updated by STS marker content and by comparison of BAC end sequences with the available draft sequence of chromosome 16q24.3 (Fig. 1A).

3. Functional analysis of BAC clones

Our mapping data revealed that four DNA makers (D16S3063, D16S3048, WI15838, and WI12410), out of six present on the complementing YAC d792t2 (Reddy et al. 2000, Oncogene 19: 217-222), were present on each of two overlapping BACs, 344A17, 346J21 (Fig.1A). These markers were deleted in the immortal revertants clones of YAC transfer senescent clones of LA.7 cells, suggesting a potential site for the SEN16 gene. Thus BACs 344A17 and 346J21, each carrying all four markers, were selected as potential candidates for functional testing. Two other BACs, 924B9 and 276J15, mapped outside YAC d792t2, were selected as
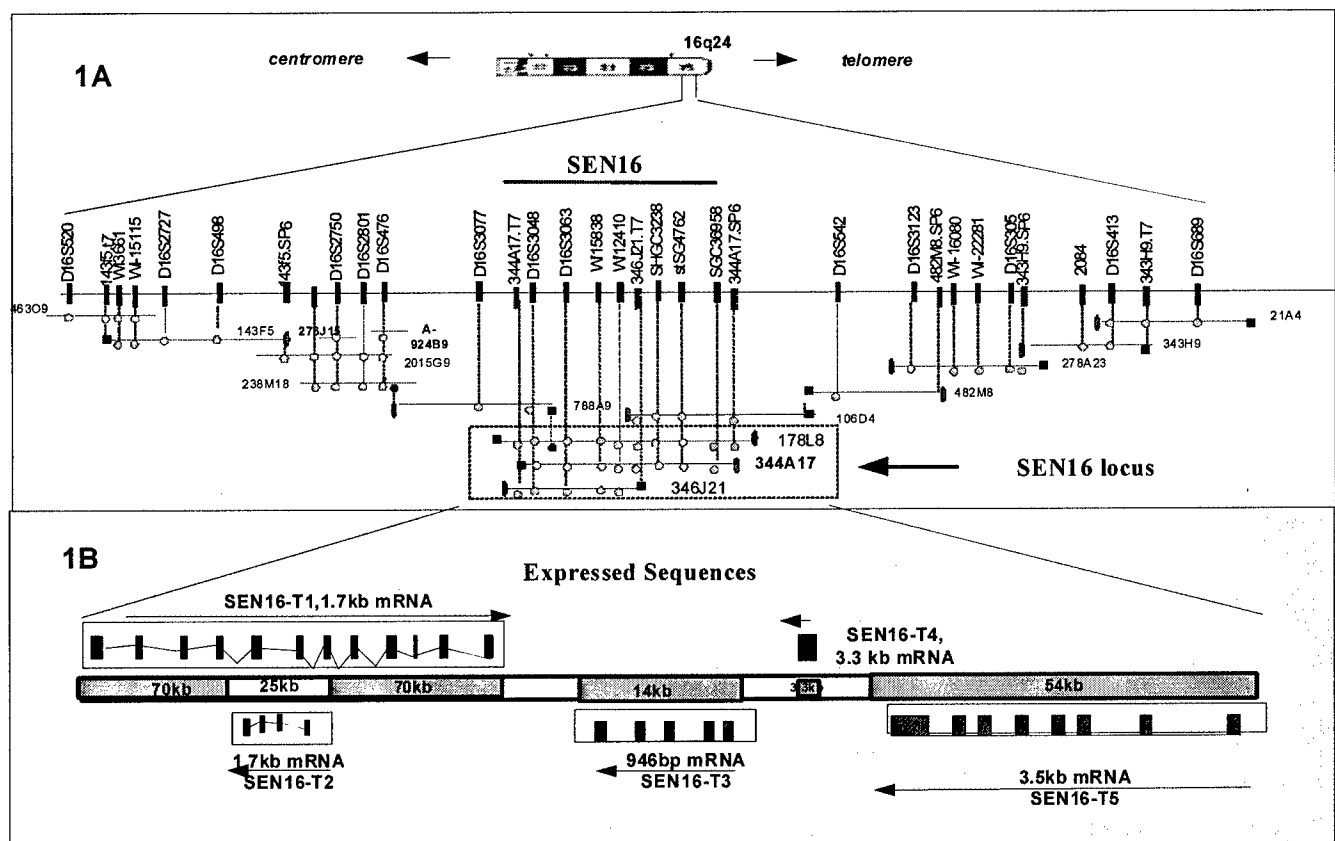

Fig.1 Physical and transcript map of SEN16 locus
A. Contig of 15 BAC clones along with mapped markers.
B. cDNAs corresponding to the transcribed sequences from SEN16 locus. These cDNA are assembled from sequence of partial EST clones, sequence of RT-PCR results and database search. Since all cDNAs are not characterized as yet, each cDNA is designated as transcriptional unit named as SEN16T1, SEN16T2, SEN16T3, SEN16T4 and SEN16T5.

controls for functional test.

BAC clones were retrofitted to incorporate a copy of the *neo* gene, a marker for selection in mammalian cells, using a vector exchange procedure(30). Retrofitted clones 346J21C5, 924B9C1 and 276J15C6, representing each respective BAC, were used in the functional assay (Table 1). The origin of human insert in the retrofitted BAC clone, 346J21C5, was confirmed to be from 16q24.3, by FISH on to the metaphase spreads, prepared from normal human lymphocytes. A single hybridization signal at 16q24.3 confirmed that 346J21 is a non-chimeric BAC.

DNA purified from each of the three retrofitted BACs was introduced into human and rat immortal tumor cell lines using lipofection and electroporation methods. Immortal recipient cell lines used for BAC transfer included MCF.7, MDA-MB468 (human breast tumor), T98G (human glioblastoma), LA7 (rat mammary tumor), and A9 (a mouse immortal cell line). Multiple independent transfection experiments were performed with each recipient cell line. G418 resistant colonies, which appeared in the subsequent three-week period, were either isolated individually into new plates or followed in the parent plates. All colonies were

continuously maintained in selection medium containing G418 and examined at regular intervals to assess colony and cell morphology and growth characteristics. Data on the number of colonies along with colony and cellular phenotypes are presented in Table 1.

Table 1: Number and phenotype of colonies recovered following BAC transfer into different immortal cell lines

| BACs | Cell Lines | # of Expts | Number of Colonies | | | | Cell Division Interval (hours)[+] | Length of survival of senescent colonies[++] (weeks) |
			Senescent	Mixed cell population	Parental Type	Total		
346J21	LA7	4	12	15	8	35	30-40	4-6
	MCF.7	4	28	38	6	72	90-96	5-7
	MDAAMB468	2	30	--	48[+++]	78	90-96	5-7
	T98G	2	0	0	35	35	20-24	imm[++++]
	A9	2	0	0	46	46	18-20	imm
924B9	LA7	4	0	0	25	25	15-18	imm
	MCF.7	4	0	0	60	60	20-24	imm
	MDAAMB468	2	0	0	45	45	20-24	imm
	T98G	2	0	0	26	26	20-24	imm
	A9	2	0	0	37	37	18-20	imm
276J15	LA7	4	0	0	30	30	15-18	imm
	MCF.7	4	0	0	39	39	20-24	imm

+ Determined by counting cells under phase contrast microscope at regular intervals of cell growth
++ Interval between time of BAC transfer and peeling off of senescent clones
+++ Represents a total of mixed cell population and parental type cells
++++ Immortal cell population

Transfer of BAC 346J21C5 into MCF.7, MDA-MB-468 and LA7 cells gave two types of colonies i.e. senescent and immortal (Table 1, Fig. 2). Cells in senescent colonies displayed an initial doubling time of 72-96 h that increased progressively until complete growth arrest. At this stage, cells became enlarged, flattened and vacuolated and each colony contained 500-1000 cells (Fig.2). Cells remained attached to plates for additional 1-2 months and were eventually stained for senescence-associated b-gal (SA b-gal) activity (Fig 2) or harvested for PCR analysis. Senescent colonies displayed characteristic peri-nuclear staining showing the expression of SA□-gal activity (Fig.2). In contrast, immortal colonies were indistinguishable from the parental cells, which multiplied indefinitely and were negative for SA-β-gal activity.

BAC 346J21C5 did not restore senescence in T98G, a human glioblastoma cell line (Table 1). In this case all BAC transfer colonies multiplied indefinitely, implying that the effect is cell line specific. This is attributed to genotypic differences in cell lines resulting in the disruption of the pathway, leading to immortal proliferation, at a different site.
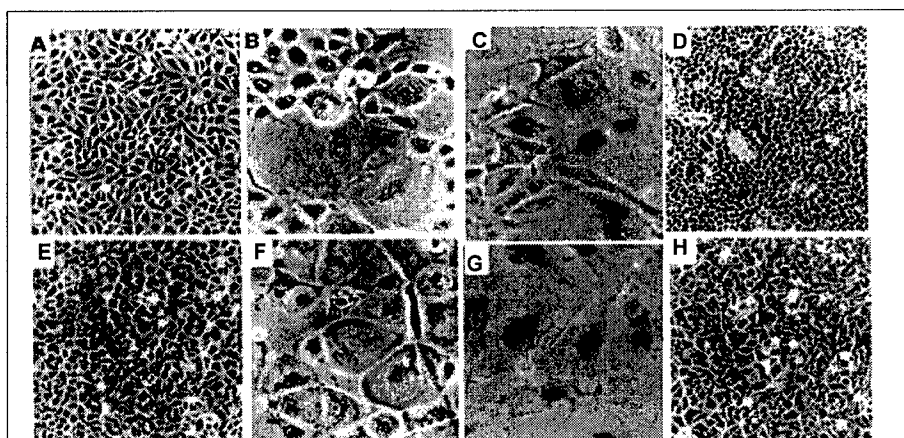

Figure 2: Photomicrographs of human MCF.7) and rat (LA7)mammary tumor cells and BAC transfer clones. (A) LA7 parental cells;(B) A senescent clone of LA7 following the transfer of BAC 346J21 showing flat and enlarged cell morphology;(C) Cells from senescent clone stained with SA-β-gal; (D) An immortal clone of LA7 with BAC 924B9 ;(E) MCF.7 cells; (F)A senescent clone of MCF.7 following the transfer of BAC 346J21, showing flat and enlarged cell morphology; (G) Cells from senescent clone stained with SA-β-gal; (H) An immortal clone of MCF.7 containing BAC 924B9 (Photomicrographs taken at same magnification).

Two types of control experiments performed to confirm our results included the transfer of empty retrofitting vector, pJMOX166, and BAC clones located at a chromosomal region away from the SEN16 locus. Introduction of pJMOX166 or BACs 924B9C1 and 276J15C6 did not restore senescence and/or normal cell growth in any of the immortal cell lines (Table 1). BAC transfer clones, in this case, were also negative for SA-ß gal activity (Fig. 2).

The immortal colonies of MCF.7, MDA-MB-468 and LA7 cells, observed following the introduction of BAC 314J21C5, occurred due to the truncation of the BAC during the process of integration into the recipient cell chromosome. In addition, during continued maintenance of senescent colonies of MCF.7, MDA-MB-468 and LA7 cells in selection medium, rare spontaneous revertant clone arose among growth-arrested cells in some of the colonies. Analysis of independent immortal colonies of LA7/346J21C5 showed the loss of all markers except D16S3063, indicative of BAC truncation. In comparison all senescent clones showed the presence of all STS markers. These data confirmed that revertant clones arose in LA7 due to the loss of donor BAC sequences and that SEN16 activity is located around the lost markers, WI15838, WI12410 and D16S3048 (Fig.1A).

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In case of human breast tumor cells, since none of the markers showed polymorphism between MCF.7, MDA-MB468 and 346J21 DNA, the retention of donor BAC alleles in immortal clones of human cells could not be assessed. However, all immortal clones of human cells were positive for neo marker showing the presence of the donor BAC segment. Presence of the donor BAC in transfer colonies of 924B9 was confirmed by the retention of D16S476, a polymorphic marker, and *neo* gene (data not shown).

In conclusion, BAC transfer experiments show that BAC 346J21 carries a replicative senescence gene that may be mutated in mammary tumors and/or tumor derived cell lines.

4.   Identification of expressed sequences from BAC 346J21

BLAST search of the human genome database, with partial nucleotide sequence of BACs 346J21 and 344A17, identified another 175 Kb BAC (178L8) that encompasses the genomic region covered by 346J21 and 344A17 (Fig. 1A). Since the complete sequence of 178L8 is available in the database, the sequence of BACs 346J21 and 344A17 was retrieved by aligning the end sequences.

BLAST analyses of the retrieved sequence of BACs 346J21 and 344A17 identified 8 ESTs located on BAC 346J21. Another set of 6 expressed sequences was recovered, from the genomic insert in BAC 346J21, by exon trapping. Partial cDNA clones, corresponding to all 14 expressed sequences, were identified in the database and obtained from commercial sources. The human DNA inserts in partial cDNA clones were fully sequenced and compared against each other to identify overlapping clones originating from the same transcripts. Each cDNA sequence was examined by BLAST search against genomic sequence to determine its location and establish exon-intron boundaries. In addition, forward and reverse PCR primers, designed corresponding to the ends of each cDNA, were used for RT-PCR to search for possible linking transcripts located between two available cDNAs. All these data were combined to assemble 5 larger cDNAs clones and construct a transcript map of SEN16 locus (Fig.1B).
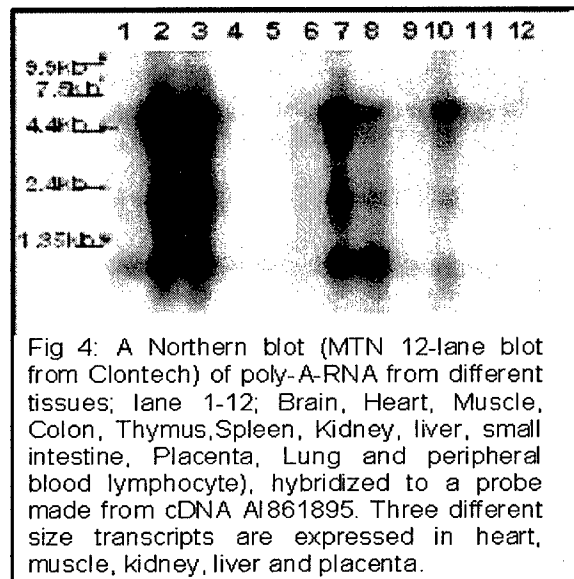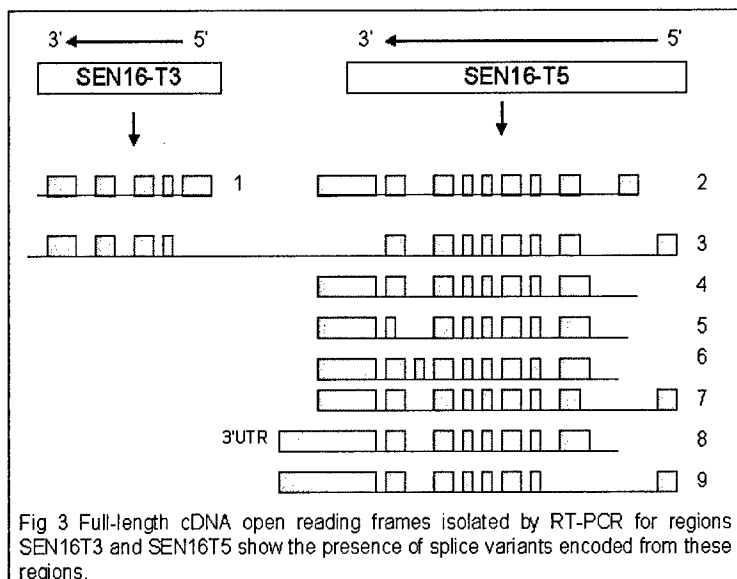
We have temporarily named these cDNAs as transcription units SEN16T1, SEN16T2, SEN16T3, SEN16T4 and SEN16T5 (Fig. 1B, T stands for transcript). While SEN16T1 is a 1.7Kb transcript that contains 5 exons and is encoded from the positive strand (i.e. 5' centromere ' to 3' telomere), SEN16T2 is 1.7 Kb in size comprised of 4 exons encoded from the same region but from the negative strand of the genomic DNA. SEN16T3 is a 0.95 Kb transcription unit with multiple splice forms, comprised of 5 exons, expressed from the negative strand. SEN16T4 is a 3.0 Kb single intron less transcript that also comes from the negative strand. Several different splice variants of SEN16T5, each comprised of different exons are expressed from the negative strand in normal and tumor cells. Some of the splice variant forms of SEN16T5 are different in tumor cells than those in normal cells. Expression of each of these transcripts has been experimentally verified by RT-PCR and sequencing analysis. The open reading frame, translation initiation and stop codon for each of the three transcripts (SEN16T1, SEN16T3, SEN16T4 and SEN16T5) were identified by computer analyses.

Primers designed from the ends of each transcript were used to search for intervening linking transcript segments. Interestingly, our investigations revealed that SEN16T3 and SEN16T5 are the part of a single gene, expressed as two independent genes as well, thus, making it as three different genes in the region. It appears to be a region of highly complex genes, with yet unknown number of splice variants. We have identified 9 different full-length ORF for the transcripts encoded from SEN16T5/T3 regions (Fig. **3**). These transcripts are differentially expressed in different tissues. At the same time, different isoforms are expressed in breast cancer cells as compared to the normal cells (data not shown).

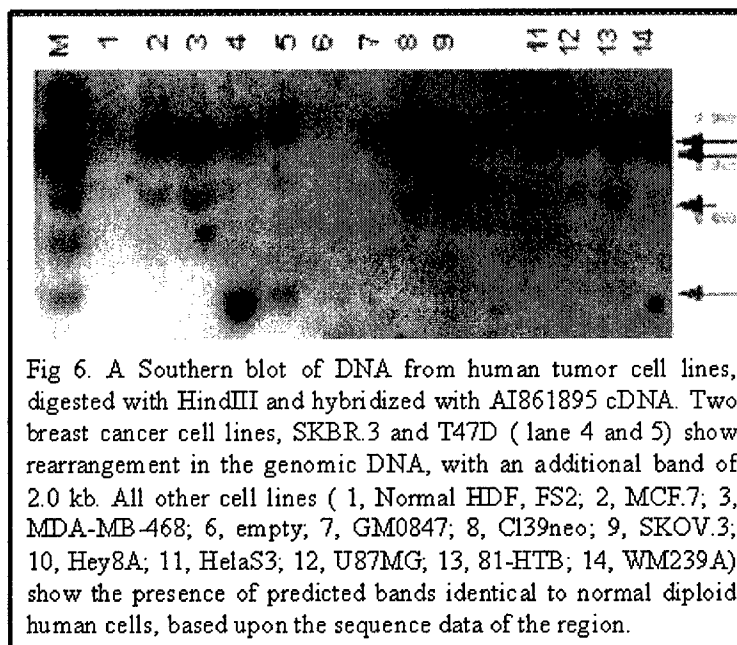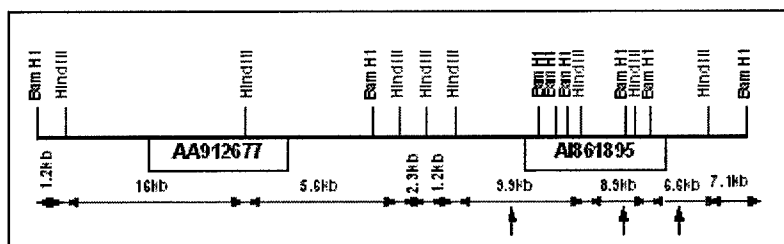## Analysis to determine the size of full-length transcripts

In order to determine the approximate size of the full-length transcripts for different cDNA clones, multi-tissue Northern blots (Clontech Inc) were probed with partial cDNA clones. Northern blot data obtained with an EST clone AI861895 (part of SEN16T3) revealed presence of multiple transcripts, varying from 1 kb to approximately 6 kb in size and differentially expressed in different tissues (Fig. 4). Also relative abundance of different size transcripts varied in different tissues (Fig. 4). These data suggested the existence of multiple splice variants for the transcript encoded from the gene represented by SEN16T3, whereas only a single transcript for

EST clone AA91277 (part of SEN16T2) was detected in testes (data not shown). These analyses are still in progress using other cDNA clones.

Fig 3 Full-length cDNA open reading frames isolated by RT-PCR for regions SEN16T3 and SEN16T5 show the presence of splice variants encoded from these regions.

Fig 4: A Northern blot (MTN 12-lane blot from Clontech) of poly-A-RNA from different tissues; lane 1-12; Brain, Heart, Muscle, Colon, Thymus,Spleen, Kidney, liver, small intestine, Placenta, Lung and peripheral blood lymphocyte), hybridized to a probe made from cDNA AI861895. Three different size transcripts are expressed in heart, muscle, kidney, liver and placenta.

5. Identification of candidate cDNAs for SEN16 gene

Next task in these studies was to determine if any of these cDNAs represents SEN16 gene. An important characteristic of a senescence/tumor suppressor gene is rearrangements or loss of expression due to mutations or epigenetic effects.

Fig 5. Restriction map of chromosomal region represented by AA912677 and AI861895 cDNAs.

a. Analysis for genomic deletions at SEN16 locus in immortal cell lines: Mutations resulting in a disease phenotype can be classified into two major categories: those that cause major changes in gene structure (large deletions, insertions, duplications and inversions) and those that cause only a minimal change in gene structure (single base changes as well as small deletions, inversions, duplications, or insertions). We applied Southern blot hybridization and PCR to look for deletions or genomic rearrangements.

We used EST clone AI861895 (part of SEN16T3) as a molecular probe for detecting deletions or rearrangements in 22 immortal tumors cell lines derived from different tumors. Since the genomic sequence of SEN16 region is known, the number and size of the expected hybridization bands can be precisely predicted in a Southern blot (Fig 5). Complete loss or altered size of the bands would indicate genomic rearrangements.

Fig 6. A Southern blot of DNA from human tumor cell lines, digested with HindIII and hybridized with AI861895 cDNA. Two breast cancer cell lines, SKBR.3 and T47D (lane 4 and 5) show rearrangement in the genomic DNA, with an additional band of 2.0 kb. All other cell lines (1, Normal HDF, FS2; 2, MCF.7; 3, MDA-MB-468; 6, empty; 7, GM0847; 8, C139neo; 9, SKOV.3; 10, Hey8A; 11, HelaS3; 12, U87MG; 13, 81-HTB; 14, WM239A) show the presence of predicted bands identical to normal diploid human cells, based upon the sequence data of the region.

Predicted hybridization bands were observed in all of the tumor cell lines except in two breast tumor cell lines

T47D and SKBR.3. These two cell lines did not show the expected size band; instead a new 2Kb band appeared, indicating genomic deletion or rearrangement. Search for rearrangements with other cDNAs is in progress.

PCR for the amplification of mapped markers was used as another approach to identify homozygous deletions in tumor cells. PCR analysis of 22 immortal cell lines, with all available markers, identified homozygous deletions (data not shown) in MCF.7 and cl39 cells, in the region of AI861895 (part of SEN16T3) and AA906579 (part of SEN16T5).

b. **Analysis for the expression of cDNA sequences in immortal cell lines**: 22 immortal cell lines, derived from different tumors or SV40 transformed human cells, were examined by RT-PCR for the loss of RNA transcripts for partial cDNAs from SEN16 locus. These data revealed the loss of expression of an EST clone AA906579 (representing SEN16T5 transcript) in two of the cell lines, MCF.7 and Cl39 (Fig 6).

In conclusion, the results of these studies revealed genomic deletions or loss of expression in some of the breast tumor cell lines. Chromosomal region 16q24.3 displays high incidence of LOH in many different cancers and has been under intense investigation for tumor suppressor genes in several different labs around the globe. The genomic rearrangements observed at the SEN16 locus, especially in the expressed sequences, in tumor cell lines, suggest a possible role for this region in the etiology of breast cancer.

6. Cloning of full-length cDNAs and functional testing:

Five different transcription units (Fig. 1B) were identified from computer analysis of human genomic sequences and from the sequence of partial cDNAs cloned by others and us. We have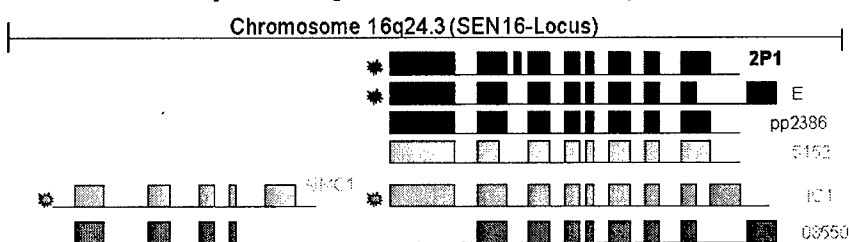 identified at least 9 full length ORF following RT-PCR cloning and sequence analysis (Fig. 3). Four of the full length cDNAs, 2P1, E, ICI and AIMC1 (Fig. 7), have been cloned into a mammalian cell expression vector pcDNA3.1, in frame with FLAG or myc tag. Cloned genes were verified for sequence integrity of the coding region and the tag. Plasmid construct AIMC1 was transferred into mammary tumor cell lines, MCF.7,

Fig: 7 Full length cDNAs (different isoforms) cloned in pcDNA 3.1 in frame with a tag (2P1-Flag , E-myc, IC1-Flag and AIMC1-Flag), these clones are being tested for function in breast cancer cell lines MCF.7, SKBR.3, MDA-MB-468 and T47D

MDA-MB468 and LA.7. G418 resistant gene transfer colonies were either followed in the parent plates or isolated individually. All colonies were examined at regular intervals to assess colony and cell morphology and growth characteristics.

Following ectopic expression of AIMC1 in MDA-MB468 cells, two types of colonies i.e. senescent and immortal were recovered. Cells in senescent colonies displayed a progressively diminished growth rate leading to complete growth arrest (Fig.8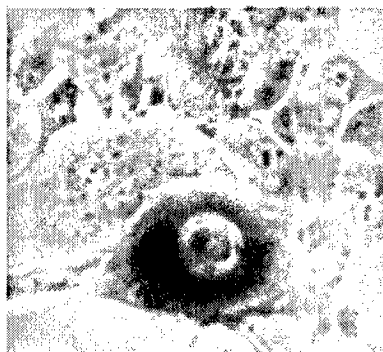). At this stage cells attached to plates were stained for senescence-associated-β-gal (SA-β-gal) activity (Fig.8). Senescent cells remain attached to the plates for long periods of time. One of the colonies is maintained in culture for longer than 6 month period, displayed senescent phenotypes but lacked DNA replication as examined by Brdu incorporation (data not shown).

Senescent colonies displayed characteristic peri-nuclear staining showing the expression of SA□-gal activity (Fig. 8). In contrast, immortal colonies were indistinguishable from the parental cells, which multiplied indefinitely and were negative for SA β-gal activity. PCR analysis of DNA isolated from individual colonies revealed that immortal colonies did not contain intact cDNA, but carried the *neo* marker. Loss of the cloned fragment could occur during the process of transfection or integration into the cell genome. In comparison, all senescent colonies analyzed, contained intact cDNA. Due to the limited

Fig 8: Photomicrograph of gene transfer clone of MCF.7 cells, showing typical characterstics of senescent cells and perinuclear SA-β-Gal activity.

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number of cells present in senescent colonies, analysis for cell cycle parameters could not be performed. Also the number of colonies recovered in vector control vs AIMC1 gene transfer was at least 50 fold more. These data revealed that AIMC1 gene, expressed from a strong CMV promoter, leads to immediate growth arrest and senescence giving reduced number of viable colonies.

Experiments with MCF.7, T47-D and LA7 cells are inconclusive. For each of these cell lines 10 independent colonies were isolated and preserved for analysis. These colonies have not yet been analyzed. We will continue these studies to investigate the effect of other genes in same cell lines.

Difficulties Encountered in these Experiments:
The results of the above experiments suggest that over expression of AIMC1 cDNA, driven by a strong generic promoter, limits the proliferation potential of the cell and induces senescence at an early stage. Thus, using pcDNA vector, it may be difficult to obtain an adequate cell population of gene transfer clones for further analyses. Also, tag protein FLAG used for detection of the AIMC1 expression binds to a non specific product in size range of expected protein product. Thus, we could not distinguish the AIMC1 product from the non specific bands in the western blots.

In order to get around these problems, we have cloned these genes into a retroviral vector (PQCXIP) in frame with EGFP tag. This will allow us to follow the gene expression by immunofluorescence and western blot analysis. A retroviral vector provides highly efficient system for rapid analysis of all different cDNAs in multiple cell lines. Although retroviral vector systems with CMV promoter will be useful for initial screening of a large number cell lines for the effect of each isoform, it may be difficult to obtain an adequate cell population of a gene transfer clone for further analyses.

In order to get around the problem of over expression induced instant growth arrest and generate adequate cell population for detailed analysis, we will clone eac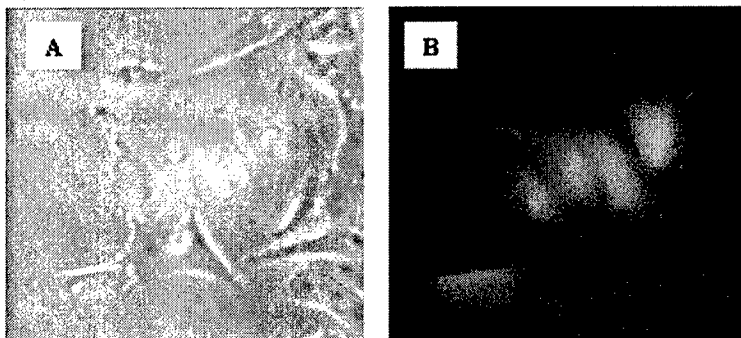h of the genes in an inducible promoter. In this case, we will have the advantage of inducing the message in the cells when required. We are using a retroviral based vector system with ecdysone-inducible promoter (Stratagene, Inc). This system is comprised of two vectors. One of the vectors pFB-ERV carries genes for retinoid X receptor (RXR), ecdysone receptors (VgEcR) and *neo* driven by CMV promoter as a single transcript. The second vector, pCFB-EGSH, which is used for cloning the gene of interest, carries ecdysone glucocoricoid response element (E/GRE) sites, located upstream to minimal

Fig 9 Stable clones of MCF7 cells expressing RXR and VgEcR, transfected with pIEGFP and induced with Muristerone A (10 uM) for 48 hour. A) Phase contrast and B) Flourescence photomicrographs.

heat shock (mHSP) promoter along with *hyg* gene for selection in mammalian cells. Each vector will be propagated in an appropriate packaging cell line and introduced into the tester cell lines through viral infection.

In the presence of inducer ecdysone or its analogs Muristerone A or Ponasterone A, RXR and VgEcR form dimers. The dimerized complex acts as a transcription factor, binds to E/GRE sites, located in the second vector upstream to the mHSP promoter, and induces the expression of the cloned gene. In the absence of the inducer, two receptors RXR and VgEcR cannot form dimers and fail to bind to E/GRE sites and the gene remains turned off. Since the system is dependent upon the expression of two receptors, it provides a non-leaky complete control of gene expression (31-32) . We have already generated clonal tester cell lines, expressing RXR and VgEcR receptors for tumor cell lines SKBR3, T47D and MCF7 (Fig 9).

Summary:
1. We have identified candidate cDNAs encoded from the genomic sequence carried in BAC 346J21 that restores senescence in MCF.7 and LA.7 cells.
2. We have constructed full length ORF for candidate cDNAs .

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3. While multiple splice variant isoforms are present in normal and tumor cells, some of the variants identified in breast tumor cells are different than those in normal cells.
4. Genomic rearrangement and lack of expression of some cDNA, detected at SEN16 locus in tumor cells, points to a role in the etiology of breast cancer.
5. Expression of a cloned cDNA, encoded from the region of genomic rearrangement, restored senescence in at least one breast tumor cell line (MDAMB-468).
6. Protein database search predicted the presence of F-box motif in two of the transcripts. Proteins containing F-box motif are known to interact with cell cycle proteins e.g. G1-Cyclins, cyclin-cdk inhibitors and transcription factors.

The results of our experiments support that we indeed may have identified a cDNA for a cell senescence gene. In future experiments we will establish the role of SEN16 in cell proliferation and in the etiology of breast cancer.

C. Key research accomplishments

1. Applying a functional approach, starting with an intact chromosome, we progressively reduced the genomic region carrying SEN16 to a 185 Kb DNA segment carried in BAC clone (346J21) that restores senescence in breast tumor cell lines MCF.7 and LA7.
2. Identified candidate cDNAs encoded from the genomic sequence in BAC 346J21.
3. We have cloned 4 different full-length cDNAs, representing 3 novel genes located at the SEN16 locus.. Each gene is cloned in a mammalian cell expression vector in frame with a Tag protein for immunological detection.
4. We have established the genomic structure of the gene, identified exon – intron boundaries for various transcript isoforms of four candidate genes.
5. Functional testing show that one of the genes, AIMC1, tested for function has been found to induce senescence in one breast cancer cell lines.
6. We have established an Ecdysone inducible expression system in retroviral vectors for the analysis of cloned genes.
7. Experiments are currently underway to clone all 4 cDNAs in inducible vector.
8. Identified chromosomal rearrangements/ deletion at SEN16 locus in breast tumor cell lines.

D. Reportable Outcome

Abstracts of Presentations:
1. Kaur G, Athwal RS and Kaur GP (2000) A functional-positional approach towards cloning a cell senescence gene at 16q24.3. Proceedings of 91st Annual AACR meeting, San Francisco, CA.
2. Kaur GP and Athwal RS (2002) Isolation of expressed sequences from a genomic segment carrying senescence gene (SEN16) for breast tumor cells. Amer. J. Hum. Genet. 71 (supplement): #419.
3. Kaur GP and Athwal RS (2003) Cloning of candidate cDNAs for a novel senescence gene in breast tumor cells. Proceedings of 94th Annual AACR meeting, Washington,DC.

Relevant publications

1. Reddy D.E., Sandhu A.K., DeRiel, J.K., Athwal, R.S. and Kaur G.P. (1999) Identification of a gene at 16q24.3 that restores cellular senescence in immortal mammary tumor cells. Oncogene 18:5100-5107

2. Reddy, D.E., Keck, C.L., Popescu, N., Athwal, R.S. and Kaur, G.P. (2000) Identification of a YAC from 16q24 carrying a senescence gene for breast cancer cells. Oncogene 19: 217-222.

3. Kaur, G.P., Reddy, D., Zimonjic D. B., Riel, J. K. and Athwal, R. S. (2004) Functional identification of a BAC clone from 16q24 carrying a senescence gene SEN16 for breast cancer cells. Oncogene 24: 47-54.

4. Two manuscripts in preparation.

E. Conclusions

We have successfully accomplished Aim 1, Aim 2 and part of Aim 3 as proposed in this project. Project is continued to establish mutation spectrum in breast tumors and tumor cell lines. Four full length candidate cDNAs have been cloned. In preliminary studies, ectopic expression of one of the cDNA clones inhibits growth in MDA-MB468 cells and induces senescence. These results point to a role of SEN16 in cell growth and senescence. We will use a retroviral vector system to evaluate the effect of different candidate cDNAs in multiple cell lines. Once a senescence gene is identified, we will use an inducible vector system for functional analysis of the gene. We have already established the inducible expression system in a retroviral vector for these studies.

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